

**Please amend the specification as follows:**

At page 107, please replace the paragraph beginning in line 15 with the following paragraph:

- - Cultures of phage transduced bacteria were prepared in 10-100 mls 2 x TY medium with 15 µg/ml tetracycline and grown with shaking at 37°C for 16-24 hrs. Phage supernatant was prepared by centrifugation of the culture (10 min at 10,000 rpm, 8 x 50 ml rotor, Sorval RC-5B centrifuge). At this stage, the phage titre was 1 - 5 x ~~10<sup>10</sup>~~ 10<sup>10</sup>/ml transducing units. The phage were precipitated by adding 1/5 volume 20% PEG 2.5 M NaCl, leaving for 1 hr at 4°C, and centrifuging (supra). The phage pellets were resuspended in 10 mM Tris-HCl, 1mM EDTA pH 8.0 to 1/100th of the original volume, and residual bacteria and aggregated phage removed by centrifugation for 2 min in a bench microcentrifuge. - -

At page 111, please replace the paragraph beginning in line 6 with the following paragraph:

- - PCR conditions were as described in example II, except that thirty cycles of PCR amplification were performed with denaturation at 92°C for 45 seconds, annealing at 55°C for 1 minute and extension at 72°C for 1 minute. The template used was DNA from TG1 cells containing Fab D1.3 in pUC19 resuspended in water and boiled. The template DNA was prepared from the colonies by picking some colony material into 100µl of distilled H<sub>2</sub>O and boiling for 10 mins. 1µl of this mixture was used in a 20µl PCR. This regime resulted in amplification of the expected fragment of approximately 600bp. This fragment was cut with Pst I and Xho I, purified from an agarose gel and ligated into Pst 1/Xho 1-cut fdCAT2. The PCR mixture was extracted with phenol/chloroform and ethanol precipitated (Sambrook et al. supra.) before digestion with Pst1 and Xho1 (New England Biolabs according to manufacturers recommendations. The

fragment was resolved on 1% Tris-Acetate EDTA agarose gel (Sambrook et al. supra) and purified using GeneClean (BIO 101, GeneClean, La Jolla, San Diego, California, USA) according to manufacturers recommendations. - -

At page 112, please replace the paragraph beginning in line 6 with the following paragraph:

- - 75ng of Pst 1/Xho 1-digested vector DNA was ligated to 40ng of PCR-amplified Pst1 /Xho I-digested hEGF-R fragment in 12µl of ligation buffer (66mM TrisHCl (pH7.6), 5mM MgCl<sub>2</sub>, 5mM dithiothreitol, (100µg/ml bovine serum albumin, 0.5mM ATP, 0.5mM Spermidine) and 400 units T4 DNA ligase (New England BioLabs) for 16 hours at 16°C. - -

At page 112, please replace the paragraph beginning in line 13 with the following paragraph:

- - Two µl of the ligation mixture was transformed into 200µl of competent E.coli MC1061 cells, plated on 2TY agar containing 15µg/ml tetracycline and incubated at 30°C for 20 hours. A portion of the ligation reaction mixture was transformed into E.coli MC1061 (Available from, for example Clontech Laboratories Inc, Palo Alto, California) and colonies identified by hybridisation with the oligonucleotide D1.3CDR3A as described in example 10. The presence of the VHCH1 gene fragment was likewise confirmed by PCR, using oligonucleotides KSJ6 and 7. A representative clone was called fd CAT2VHCH1 D1.3. The heavy chain was deleted from Fab D1.3 in pUC19 by Sph I cleavage of Fab D1.3 plasmid DNA. The pUC 19 2.7Kb fragment containing the light chain gene was purified from a TAE agarose gel, and 10ng of this DNA self-ligated and transformed into competent E.coli TG1. Cells were plated on 2TY agar containing ampicillin (100µg/ml) and incubated at 30°C overnight. The resulting colonies were used to make miniprep DNA (Sambrook et al. supra), and

the absence of the heavy chain gene confirmed by digestion with Sph I and Hind

III. A representative clone was called LCD1.3 DHC. - -

At page 113, please replace the paragraph beginning in line 9 with the following paragraph:

- - An overnight culture of fd CAT2VHCH1 D1.3 cells was microcentrifuged at 13,000Xg for 10 minutes and 50µl of the supernatant containing phage particles added to 50µl of an overnight culture of LCD1.3 DHC cells. The cells were incubated at 37°C for 10 minutes and plated on 2TY agar containing ampicillin (100µg/ml) and 15µg/ml tetracycline. Phage were prepared from some of the resulting colonies and assayed for their ability to bind lysozyme as described in example 6. - -

At page 116, please replace the paragraph beginning in line 3 with the following paragraph:

- - 40 pmole of oligonucleotide VH1FOR (Ward, E. S., et al (1989) Nature 341, 544-546), specific to pAb (D1.3) was phosphorylated with 100 µCi α-32P ATP, hybridised (1pmole/ml) to nitrocellulose filters at 67°C in 6 x saline sodium citrate (SSC) Sambrook et al., supra. buffer for 30 minutes and allowed to cool to room temperature for 30 mins, washed 3 x 1 min at 60°C in 0.1 x SSC. - -

At page 116, please replace the paragraph beginning in line 27 with the following paragraph:

- - The specific binding of pAb NQ11 was confirmed using ELISA. ELISA plates were coated at 37°C in 50 mM NaHCO<sub>3</sub> at a protein concentration of 200 µg/ml. Plates were coated with either hen egg lysozyme (HEL), bovine serum albumin (BSA), or BSA conjugated to oxazolone (OX-BSA) (method of conjugation in Makela O., Kartinen M., Pelkonen J.L.T., Karjalainen K. (1978) J. Exp. Med. 148 1644). Preparation of phage, binding to ELISA plates, washing and detection was as described in example 6. Samples were assayed in duplicate and the average absorbance after 10 minutes presented in figure 14.

This result demonstrates that the pAb NQ11 binds the correct antigen. Figure 14 also shows that pAb D1.3 and pAb NQ11 bind only to the antigen against which the original antibodies were raised.

At page 118, please replace the paragraph beginning in line 26 and continued to page 119 with the following paragraph:

- - The PCR reaction was carried out in 100µl of 10 mM Tris/HCl pH 8.3, containing 50 mM KCl, 5mM dNTP 2.5 mM MgCl<sub>2</sub>, 0.01% gelatin, 0.25 units/µl of Taq polymerase (Cetus/Perkin Elmer) and 0.5µg/ml template. The template was the pEK86 plasmid (described by Chaidaroglou et al., Biochemistry 27 p8338-8343, 1988). The PCR was carried out in a Techne (Techne, Duxford, Cambridge, UK) PHC-2 dri-block using thirty cycles of 1 min at 92°C, 2 min at 50°C, 3 min at 72°C. - -

At page 119, please replace the paragraph beginning in line 8 with the following paragraph:

- - The resultant product was extracted with phenol:chloroform, precipitated with ethanol, and the pellet dissolved in 35µl water. Digestion with 0.3 units/µl of Apa L1 was carried out in 150µl volume according to manufacturers instructions for two hours at 37°C. After heat inactivation of the enzyme at 65°C, NaCl was added to a final concentration of 150mM and 0.4 units/µl Not1 enzyme added. After incubation for 2 hours at 37°C, the digest was extracted with phenol:chloroform and precipitated as above, before being dissolved in 30µl of water. The vector fd-CAT2 was sequentially digested with Apa L1 and Not1 according to the manufacturers instructions and treated with calf intestinal alkaline phosphatase as described in example 2. The sample was extracted three times with phenol:chloroform, precipitated with ethanol and dissolved in water. The ligations were performed with a final DNA concentration of 1-2ng/µl of both the cut fd-CAT2 and the digested PCR product. The ligations were transformed

into competent TG1 cells and plated on 2xTY tet plates. Identification of clones containing the desired insert was by analytical PCR performed using the conditions and primers above, on boiled samples of the resulting colonies. The correct clone containing the phoA gene fused in frame to gene III was called fd-phoAla 166. The sequence at the junction of the cloning region is given in figure 15. - -

At page 120, please replace the paragraph beginning in line 10 with the following paragraph:

- - Overnight cultures of TG1 or KS272 (E.coli cells lacking phoA. Strauch K. L., and Beckwith J. PNAS 85 1576-1580, 1988) cells containing either fd-phoAla 166 or fd-CAT2 were grown at  $37^{\circ}\text{C}$  in 2xTY with  $15\mu\text{g/ml}$  tetracycline. Concentrated, PEG precipitated phages were prepared as described earlier. Enzyme assays (Malamy, M.H. and Horecker B.L., Biochemistry 3, p1893-1897, (1964)) were carried out at  $24^{\circ}\text{C}$  in a final concentration of 1M Tris/HCl pH 8.0, 1mM 4-nitrophenyl phosphate (Sigma), 1mM  $\text{MgCl}_2$ . 100 $\mu\text{l}$  of a two times concentrate of this reaction mixture was mixed with 100 $\mu\text{l}$  of the test sample in a 96 well plate. Absorbance readings were taken every minute for 30 minutes at a wavelength of 405nm in a Titretek Mk 2 plate reader. Initial reaction rates were calculated from the rate of change of absorbance using a molar absorbance of 17000 l/mol/cm. - -

At page 123, please replace the paragraph beginning in line 10 with the following paragraph:

- - The PCR reaction was carried out in an 80  $\mu\text{l}$  reaction as described in example 11 ~~11~~ using 1ng/ $\mu\text{l}$  of template and 0.25U/ $\mu\text{l}$  of Taq polymerase and a cycle regime of  $94^{\circ}\text{C}$  for 1 minute,  $60^{\circ}\text{C}$  for 1 minute and  $70^{\circ}\text{C}$  for 2 minutes over 30 cycles. The template was either pscFvNQ11 (example 9) or scFvD1.3 myc (example 2). Reaction products were extracted with

phenol:chloroform, precipitated, dissolved in water and digested with BamHI according to manufacturers instructions. The digest was re-extracted with phenol: chloroform, precipitated and dissolved in water. - -

At page 128, please replace the paragraph beginning in line 14 with the following paragraph:

1. Harvest  $1$  to  $5 \times 10^7$  cells by centrifugation in a bench top centrifuge at  $800 \times g$  for 10 minutes at  $4^\circ\text{C}$ . Resuspend gently in 50ml of cold PBS buffer. Centrifuge the cells again at  $800 \times g$  for 10 minutes at  $4^\circ\text{C}$ , and discard supernatant.
2. On ice, add 1 ml ice-cold lysis buffer to the pellet and resuspend it with a 1ml Gilson pipette by gently pipetting up and down. Leave on ice for 5 minutes.
3. After lysis, remove cell debris by centrifuging at 1300 rpm for 5 minutes in a microfuge at  $4^\circ\text{C}$ , in precooled tubes.
4. Transfer 0.5 ml of the supernatant to each of two eppendorfs containing 60 $\mu$ l 10% (w/v) SDS and 250  $\mu$ l phenol (previously equilibrated with 100 mM Tris-HCl pH 8.0). Vortex hard for 2 minutes, then microfuge (13000 rpm) for five minutes at room temperature. Transfer the upper, aqueous, phase to a fresh tube.
5. Re-extract the aqueous upper phase five times with 0.5 ml of phenol.
6. Precipitate with 1/10 volume 3M sodium acetate and 2.5 volumes ethanol at  $20^\circ\text{C}$  overnight or dry ice-isopropanol for 30 minutes.

7. Wash the RNA pellet and resuspended in 50  $\mu$ l to check concentration by OD260 and check 2  $\mu$ g on a 1% agarose gel. 40 $\mu$ g of RNA was obtained from spleen cells derived from mice.

At page 130, please replace the paragraph beginning in line 16 with the following paragraph:

2. Dilute 10  $\mu$ g RNA to 40  $\mu$ l final volume with DEPC-treated water. Heat at 65 $\pm$ 1 $^{\circ}$ C for 3 minutes and hold on ice for one minute (to remove secondary structure).
3. Add to the RNA the reverse transcription mix (58  $\mu$ l) and 4  $\mu$ l of the cloned reverse transcriptase 'Super RT' (Anglian Biotech Ltd., Whitehall House, Whitehall Road, Colchester, Essex) and incubate at 42 $\pm$ 1 $^{\circ}$ C for one hour.
4. Boil the reaction mix for three minutes, cool on ice for one minute and then spin in a microfuge to pellet debris. Transfer the supernatant to a new tube. 10 x first strand buffer is [1.4M KCl, 0.5M Tris-HCl pH 8.1 at 42 $\pm$ 1 $^{\circ}$ C 80mM MgCl<sub>2</sub>].

At page 132, please replace the paragraph beginning in line 10 with the following paragraph:

- - UV this mix 5 minutes. Add 2.5  $\mu$ l cDNA preparation (from B above), 2 drops paraffin oil (Sigma Chemicals, Poole, Dorset, UK). Place on a cycling heating block, e.g. PHC-2 manufactured by Techne Ltd. Duxford UK, pre-set at 94 $\pm$ 1 $^{\circ}$ C. Add 1 $\mu$ l Vent DNA polymerase under the paraffin. Amplify using 25 cycles of 94 $\pm$ 1 $^{\circ}$ C 1 min, 72 $\pm$ 1 $^{\circ}$ C 2 min. Post-treat at 60 $\pm$ 1 $^{\circ}$ C for 5 min. - -

At page 133, please replace the paragraph beginning in line 6 with the following paragraph:

- - The FOR and BACK primers are given in the section below entitled 'Primer Sequences'. The FOR primer is LINKFOR and the BACK primer is

LINKBACK. Cover with paraffin and place on the cycling heating block (see above) at  $94^{\circ}\text{C}$  1 min,  $65^{\circ}\text{C}$  1 min,  $72^{\circ}\text{C}$  2 min. Post-treat at  $60^{\circ}\text{C}$  for 5 min. - -

At page 134, please replace the first two paragraphs of the page with the following two paragraphs.

- - UV irradiate this mix for 5 min. Add  $5\mu\text{l}$  each of Vh and VK band from the primary PCRs and  $1.5\mu\text{l}$  of linker as isolated from the preparative gels and extracted using the GeneClean kit as described in C and D above. Cover with paraffin. Place on the cycling heating block preset at  $94^{\circ}\text{C}$ . Add  $1\mu\text{l}$  Vent under the paraffin. Amplify using 7 cycles of  $94^{\circ}\text{C}$  2 min,  $72^{\circ}\text{C}$  4 min. Then return the temperature to  $94^{\circ}\text{C}$ .

Add  $1.5\mu\text{l}$  each of VH1BACK and the appropriate VKFOR primers MJK1FONX, MJK2FONX, MJK4FONX or MJK5FONX ( $10\text{ pmol}/\mu\text{l}$ ) at  $94^{\circ}\text{C}$ . The primers should have been UV-treated as above. Amplify using 20 cycles of  $94^{\circ}\text{C}$  1.5 min,  $72^{\circ}\text{C}$  2.5 min. Post-treat at  $60^{\circ}\text{C}$  for 5 min. Purify on 2% 1mp/TAE gel and extract the DNA to  $20\mu\text{l}$   $\text{H}_2\text{O}$  per assembly PCR using a GeneClean kit (see earlier) in accordance with the manufacturers instructions.

At page 135, please replace the two paragraphs beginning in line 8 with the following two paragraphs.

- - Cover with paraffin and place on the cycling heating block preset at  $94^{\circ}\text{C}$ . Add  $0.5\mu\text{l}$  Cetus Taq DNA polymerase (Cetus/perkin-Elmer, Beaconsfield, Bucks, UK) under the paraffin. Amplification is carried out using 11 to 15 rounds of cycling (depends on efficiency) at  $94^{\circ}\text{C}$  1 min,  $55^{\circ}\text{C}$  1 min,  $72^{\circ}\text{C}$  2 min. Post-treat at  $60^{\circ}\text{C}$  for 5 min.

10 x Taq buffer is [ $0.1\text{M}$  Tris-HCl pH 8.3 at  $25^{\circ}\text{C}$ ,  $0.5\text{M}$  KCl,  $15\text{mM}$   $\text{MgCl}_2$ ,  $1\text{mg}/\text{ml}$  gelatin].



G. Work-up

Purify once with  $\text{CHCl}_3/\text{IAA}$  (isoamylalcohol), once with phenol, once with  $\text{CHCl}_3/\text{IAA}$  and back-extract everything to ensure minimal losses.

Precipitate and wash twice in 70% EtOH. Dissolve in 70 $\mu\text{l}$   $\text{H}_2\text{O}$ .

Digest overnight at 37°C	<u>  </u> $\mu\text{l}$
DNA (joined seq)	70
NEB NotI buffer x 10	10
NEB BSA x 10	10
NotI (10 U/ $\mu\text{l}$ )	10

At page 136, please replace the paragraph beginning in line 17 with the following paragraph.

- - Add the enzyme ApaL1 in aliquots throughout the day, as it has a short half-life at 37°C. - -

At page 139, please replace the paragraph beginning in line 9 with the following paragraph.

- - PCR amplification was performed using high fidelity conditions (Eckert, K.A. and Kunkel, T.A. 1990 Nucl Acids Research 18 3739-3744). The PCR mixture contained: 20mM TrisHCl (pH7.3 at 70°C, 50mM KCl, 4mM magnesium chloride, 0.01% gelatin, 1mM each of dATP, dCTP, dGTP and dTTP, 500ng/ml RP41 DNA, 1 $\mu\text{M}$  each primer and 50 units/ml Taq polymerase (Cetus/Perkin Elmer, Beaconsfield, Bucks, U.K.). Thirty cycles of PCR were performed with denaturation at 92°C for 1 min, annealing at 60°C for 1min and extension at 72°C for 1.5 min. This reaction resulted in amplification of a fragment of ca. 1500bp as expected. - -

At page 141, please replace the paragraph beginning in line 4 with the following paragraph.

- - Duplicate samples of 35µl concentrated phage were incubated with  $^{125}\text{I}$ -PDGF-BB (78.7fmol, 70nCi, 882Ci/mmol; Amersham International plc, Amersham, Bucks) for 1 hour at  $37^\circ\text{C}$ . Controls were included in which fdTPs/Bs vector phage (figure 4) or no phage replaced fd h-BDGF-B-R phage. After this incubation, 10µl of sheep anti-M13 polyclonal antiserum (a gift from M. Hobart) was added and incubation continued for 30 min at  $20^\circ\text{C}$ . To each sample, 40µl (20µl packed volume) of protein G Sepharose Fast Flow (Pharmacia, Milton Keynes) equilibrated in PDGF binding buffer was added. Incubation was continued for 30 min at  $20^\circ\text{C}$  with mixing by end over end inversion on a rotating mixer. The affinity matrix was spun down in a microcentrifuge for 2 min and the supernatant removed by aspiration. Non-specifically bound  $^{125}\text{I}$ -PDGF-BB was removed by resuspension of the pellet in 0.5ml PDGF binding buffer, mixing by rotation for 5 min, centrifugation and aspiration of the supernatant, followed by two further washes with 0.5ml 0.1% BSA, 0.2% Triton-X-100. The pellet finally obtained was resuspended in 100µl PDGF binding buffer and counted in a Packard gamma counter. For displacement studies, unlabelled PDGF-BB (Amersham International) was added to the stated concentration for the incubation of  $^{125}\text{I}$ -PDGF-BB with phage.

At page 144, please replace the paragraph beginning in line 8 with the following paragraph.

- - Primer A anneals to the 5' end of gene III including the ribosome binding site is located and incorporates a Hind III site. Primer B anneals to the 3' end of gene III at the C-terminus and incorporates two UAA stop codons and an EcoR1 site. 100 ng of fd-CAT2 and fd-CAT2 scFv D1.3 DNA was used as templates for PCR-amplification in a total reaction volume of 50µl as described in example 7, except that 20 cycles of amplification were performed:  $94^\circ\text{C}$  1 minute,  $50^\circ\text{C}$

0C 1 minute, 72 $\pm$ 0C 3 minutes. This resulted in amplification of the expected 1.2Kb fragment from fd-CAT2 and a 1.8Kb fragment from fd-CAT2 scFv D1.3. -

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At page 145, please replace the paragraph beginning in line 5 with the following paragraph.

- - Single pCAT-3 and pCAT-3 scFv D1.3 colonies were picked into 1.5ml 2TY containing 100 $\mu$ g/ml ampicillin and 2% glucose, and grown 6 hrs at 30 $\pm$ 0C. 30 $\mu$ l of these stationary cells were added to 6mls 2YT containing 100 $\mu$ g/ml ampicillin and 2% glucose in 50ml polypropylene tubes (Falcon, Becton Dickinson Labware, 1950 Williams Drive, Oxnard, CA. USA) and grown for 1.5 hrs at 30 $\pm$ 0C at 380rpm in a New Brunswick Orbital Shaker (New Brunswick Scientific Ltd., Edison House 163 Dixons Hill road, North Mimms, Hatfield, UK). Cells were pelleted by centrifugation at 5,000g for 25 minutes and the tubes drained on tissue paper. The cell pellets were then suspended in 6mls 2TY containing 1.25x10<sup>9</sup> p.f.u. ml<sup>-1</sup> M13KO7 bacteriophage added. The mixture was left on ice for 5 minutes followed by growth at 35 $\pm$ 0C for 45 minutes at 450 rpm. A cocktail was then added containing 4 $\mu$ l 100 $\mu$ g/ml ampicillin, 0.5 $\mu$ l 0.1M IPTG and 50 $\mu$ l 10mg/ml kanamycin, and the cultures grown overnight at 35 $\pm$ 0C, 450 rpm. - -

At page 145, please replace the paragraph beginning in line 25 and continued to page 146 with the following paragraph.

- - The following day the cultures were centrifuged and phage particles PEG precipitated as described in example 6. Phage pellets were resuspended in 100 $\mu$ l TE (tris-EDTA see example 6) and phage titred on E.coli TG1. Aliquots of infected cells were plated on 2TY containing either 100 $\mu$ g/ml ampicillin to select

for pUC119 phage particles, or 50µg/ml kanamycin to select for the M13 KO7 helper phage. Plates were incubated overnight at 37°C and antibiotic-resistant colonies counted:- -

At page 148, please replace the paragraph beginning in line 16 with the following paragraph.

- - pUC 19, pCAT-3 and pCAT-3 scFv D1.3 plasmid DNAs, and fdCAT-2 phage DNA was prepared, and used to transform E.coli TG1, pCAT-3 and pCAT-3 scFv D1.3 transformations were plated on SOB agar containing 100µg/ml ampicillin and 2% glucose, and incubated overnight at 30°C. fdCAT-2 transformations were plated on TY agar containing 15µg/ml tetracycline and incubated overnight at 37°C. Transformation efficiencies are expressed as colonies per µg of input DNA.- -

At page 151, please replace the paragraph beginning in line 23 and continued to page 152.

- - Screening for binding of the phage to hapten was carried out by ELISA: 96-well plates were coated with 10 µg/ml phOx-BSA or 10 µg/ml BSA in phosphate-buffered saline (PBS) overnight at room temperature. Colonies of phage-transduced bacteria were inoculated into 200 µl 2 x TY with 12.5 µg/ml tetracycline in 96-well plates ('cell wells', Nuclon) and grown with shaking (300 rpm) for 24 hours at 37°C. At this stage cultures were saturated and phage titres were reproducible ( $10^{10}$  TU/ml). 50 µl phage supernatant, mixed with 50 µl PBS containing 4% skimmed milk powder, was then added to the coated plates. Further details as in example 9. - -

At page 152, please replace the paragraph beginning in line 9 and continued to page 153 with the following paragraph.

- - The library of phages was passed down a phOx affinity column (Table 4A), and eluted with hapten. Colonies from the library prepared in example 22 were scraped into 50ml 2 x TY medium<sup>37</sup> and shaken at 37°C for 30 min. Liberated phage were precipitated twice with polyethylene glycol and resuspended to 10<sup>12</sup> TU (transducing units)/ml in water (titred as in example 8). For affinity selection, a 1 ml column of phOx-BSA-Sepharose (O. Makela, M. Kaartinen, J.L.T. Pelonen and K. Karjalainen J. Exp. Med. 148 1644-1660, 1978) was washed with 300 ml phosphate-buffered saline (PBS), and 20 ml PBS containing 2% skimmed milk powder (MPBS). 10<sup>12</sup> TU phage were loaded in 10 ml MPBS, washed with 10 ml MPBS and finally 200 ml PBS. The bound phage were eluted with 5 ml 1 mM 4-ε-amino-caproic acid methylene 2-phenyl-oxazol-5-one (phOx-CAP; O. Makela et al. 1978, supra). About 10<sup>6</sup> TU eluted phage were amplified by infecting 1 ml log phase E.coli TG1 and plating as above. For a further round of selection, colonies were scraped into 10 ml 2 x TY medium and then processed as above. Of the eluted clones, 13% were found to bind to phOx after the first round selection, and ranged from poor to strong binding in ELISA. -

At page 164, please replace the paragraph beginning in line 16 and continued to page 165 with the following paragraph.

- - Constructs I-IV (figure 27) were introduced into both fd-CAT2 and pHEN1. Phage fd-CAT2 (and fd-CAT2-I,II,III or IV) was taken from the supernatant of infected E.coli TG1 after shaking at 37°C overnight in 2xTY medium with 12.5µg/ml tetracycline, and used directly in ELISA. Phagemid pHEN1 (and pHEN1-I and II) in E.coli TG1 (supE) were grown overnight in 2 ml 2xTY medium, 100 µg/ml ampicillin, and 1% glucose (without glucose, expression of

g3p prevents later superinfection by helper phage). 10µl of the overnight culture was used to inoculate 2 ml of 2xTY medium, 100µg/ml ampicillin, 1% glucose, and shaken at 37°C for 1 hour. The cells were washed and resuspended in 2xTY, 100 µg/ml ampicillin, and phagemid particles rescued by adding 2 µl (10<sup>8</sup>pfu) VCSM13 helper phage (Stratagene). After growth for one hour, 4µl kanamycin (25 mg/ml) was added, and the culture grown overnight. The phagemid particles were concentrated 10-fold for ELISA by precipitation with polyethylene glycol. - -

At page 172, please replace the paragraph beginning in line 20 and continued to page 173 with the following paragraph.

- - E.coli HB2151 was infected with pHEN phagemid (pHEN1-I or II), and plated on YTE, 100µg/ml ampicillin plates. Colonies were shaken at 37°C in 2xTY medium, 100 µg/ml ampicillin, 1% glucose to OD<sub>550</sub>=0.5 to 1.0. Cells were pelleted, washed once in 2xTY medium, resuspended in medium with 100 µg/ml ampicillin, 1 mM isopropyl β-D-thiogalactoside (IPTG), and grown for a further 16 hours. Cells were pelleted and the supernatant, containing the secreted chains, used directly in ELISA. - -

At page 177, please replace the paragraph beginning in line 8 with the following paragraph.

- - 3. Centrifugation was carried out at 4°C in a microfuge at 13000 rpm for 5 min.

At page 179, please replace the paragraph beginning in line 10 and continued to page 180 with the following paragraph.

- - A total of 15ug of CsC1 purified fd-CAT2 DNA was digested with 100 units of the restriction enzyme Not I (New England Biolabs) in a total volume of 200ul 1X NEB Not I buffer with 1X NEB acetylated BSA for a total of 3 hours at 37°C. The vector DNA was the treated twice with 15ul Strataclean (a commercially

available resin for the removal of protein), following the manufacturers instructions (Stratagene, 11099 North Torrey Pines Road, La Jolla, California, USA). The DNA was then ethanol precipitated and redissolved in TE buffer (Sambrook et al., 1989 supra). The DNA was then digested with 100 units of the restriction enzyme Apa LI (New England Biolabs) in a total volume of 200ul 1X NEB Buffer 4 overnight at 37°C. The vector was then purified with a Chroma Spin 1000 column following the manufacturers instructions (Clontech Laboratories Inc, 4030 Fabian way, Palo Alto, California, USA). This step removes the Apa LI/Not I fragment to give cut vector DNA for maximum ligation efficiency. - -

At page 180, please replace the paragraph beginning in line 16 and continued to page 181 with the following paragraph.

- - Single well isolated colonies were then inoculated into 10 ml of LBtet broth (LB medium with 15ug/ul of tetracycline) in 50 ml tubes. After overnight growth at 35°C/350rpm in a bench top centrifuge. The supernatants were transferred to 15 ml centrifuge tubes and 2ml 20% PEG 8000/2.5M NaCl added to each. After incubating at room temperature for 20-30 minutes, the recombinant phage was pelleted by centrifugation at 9000rpm in a Sorval SM24 rotor for 30 minutes. The PEG supernatant was discarded. Any remaining PEG was removed with a pasteur pipette after a brief (2 minutes) centrifugation step. This last step was repeated to make sure that no PEG remained. The phage pellet was then resuspended in 500ul PBS buffer. This was transferred to a microcentrifuge tube and spun at 13000 rpm to remove any remaining cells. The phage supernatant was transferred to a fresh tube. - -

At page 182, please replace the paragraph beginning in line 13 and continued to page 183 with the following paragraph.

- - The kinetic parameters of alkaline phosphatase expressed on the surface of fd phage were investigated in 1M Tris/HCl, pH8.0 at 20°C with 1ml 4-nitrophenyl phosphate as substrate. The reactions were initiated by the addition of 100µl of a phage-alkaline phosphatase fusion preparation, 50 fold concentrated with respect to the original culture supernatant. The rate of change of absorbance was monitored at 410nm using a Philips 8730 spectrophotometer and the initial reaction rate calculated using a molar absorbance of 16200 1/mol/cm. For the fdphoAla 166 enzyme but not fdphoArg166 a lag phase was seen following this addition, the reaction rate accelerating until a steady state was obtained after approximately 60 to 90 secs. This steady state rate was used for determination of kinetic parameters. No deviation from Michaelis Menten kinetics was apparent for either phage enzyme. Values of  $K_m$  and  $k_{cat}$  were derived from plots of  $s/v$  against  $s$  and are shown in Table 6. - -

At page 185, please replace the paragraph beginning in line 26 and continued to page 186 with the following paragraph.

- - E.coli TG1 or KS272 cells (cells with a deletion in the endogenous phoA gene, Strauch and Beckwith, 1988 Supra) containing either fd-phoAla166, fdphoArg166 or fd-CAT2 were grown for 16 hours at 37°C in 2xTY with 15µg/ml tetracycline. Concentrated phage were prepared as follows. Phage-enzyme cultures are clarified by centrifugation (15 min at 10,000 rpm, 8 x 50 ml rotor, sorval RC-5B centrifuge). Phage are precipitated by adding 1/5 volume 20% polyethylene glycol, 2.5 M NaCl, leaving for 1 hr at 4°C, and centrifuging (as above). Phage pellets are resuspended in 10 mM Tris-HCl, pH 8.0 to 1/100th of



the original volume, and residual bacteria and aggregated phage removed by centrifugation for 10 to 15 minutes in a bench microcentrifuge at 13000 rpm at 4°C. - -

At page 204, please replace the two paragraphs beginning in line 3 with the following two paragraphs.

- - The reaction is decontaminated by UV irradiation to destroy foreign DNA for 5 minutes, and 1 µl of plasmid DNA added (0.1 µg/µl). The pcr mixture was covered with 2 drops of paraffin oil, and placed on the pcr block at 94°C for 5 minutes before the addition of 0.5 µl of Taq DNA polymerase under the paraffin. The cycling conditions used were 94°C 1 min, 40°C 1 min, 72°C 1.5 min 17 cycles.

The linker (Gly<sub>4</sub>-Ser)<sub>3</sub>, was amplified from the anti-phOx (2-phenyloxazol-5-one) clone fd-CAT2-scFv NQ11, using the oligos HD13BLIN and HD13FLIN3, with 0.1µg of plasmid DNA. The PCR cycling used was 94°C 1 min, 25°C 1.5 min, for 17 cycles. - -

At page 205, please replace the three paragraphs beginning in line 5 with the following three paragraphs.

- - Once again, the reaction is decontaminate by UV treatment for 5 minutes before the addition of 1 µl of the primary PCR products; VH-1 or VH-2, VK-3 or VK-4, plus the linker DNA. The reaction was covered with 2 drops of paraffin, and heated at 94°C for 5 minutes before the addition of 0.5 µl of Taq Polymerase. The PCR cycling conditions used were 94°C 1 min, 60°C 1.5 min, 72°C 2.5 min for 20 cycles.

The aqueous layer under the paraffin was extracted once with phenol, once with phenol: chloroform, once with ether, ethanol precipitated, and resuspended in 36 µl of water. To this was added, 5 µl of 10x Buffer for NotI, 5 µl 1 mg/ml

BSA, and 4  $\mu$ l (40 U) of NotI (New England Biolabs). The restriction was incubated at 37°C overnight.

The DNA was ethanol precipitated and resuspended in 36  $\mu$ l of water, and 5  $\mu$ l 10x NEB Buffer 4, 5  $\mu$ l 1 mg/ml BSA, and 2  $\mu$ l (40 U) of ApaLI (New England Biolabs). This was incubated at 37°C for 5 hours; a further 2  $\mu$ l of ApaLI was added and the reaction incubated at 37°C overnight. - -

At page 208, please replace the paragraph beginning in line 20 and continued to page 209 with the following paragraph.

- - Circles of nitrocellulose (Schleicher & Schuell, BA 85, 0.45  $\mu$ m) were labelled in pencil and lowered gently onto the colonies derived from the panning experiments and left for one minute. The filters were then pulled off quickly from one edge and placed colony side up on a piece of 3MM paper (Whatman) soaked in Denaturing solution (500 mM Sodium Hydroxide; 1.5 M Sodium Chloride) for 5 minutes. They were then transferred to 3MM soaked in Neutralizing Solution (3.0 M Sodium Chloride; 500 mM Tris-HCl, pH 7.5) for 1 minute, and then to 3MM soaked in 5x SSC; 250 mM Ammonium Acetate for 1 minute. The filters were then air dried before baking in an 80°C vacuum oven for 30 minutes. - -

At page 209, please replace the two paragraphs beginning in line 6 and continued to page 210 with the following two paragraphs.

- - The oligonucleotide probe was prepared by combining the following:

2  $\mu$ l oligonucleotide (1 pmoles/ $\mu$ l)

2  $\mu$ l  $\gamma$ -32P ATP (3000 Ci/mmol) (Amersham International plc)

2  $\mu$ l 10 x Kinase buffer (0.5 M Tris-HCl, pH 7.5; 100 mM Magnesium Chloride;

10 mM DTT)

12  $\mu$ l Water

2  $\mu$ l Polynucleotide Kinase (20 Units)

This was incubated at 37°C for 1 hour.

Hybridization was performed in the Techne HB-1 Hybridiser. The baked filters were pre-hybridized at  $37^{\circ}\text{C}$  in 40 ml of Hybridization Buffer (10 ml 100 mM Sodium pyrophosphate; 180 ml 5.0 M Sodium chloride; 20 ml 50x Denharts Solution; 90 ml 1.0 M Tris-HCl, pH 7.5; 24 ml 250 mM EDTA; 50 ml 10% NP40; made to 1 litre with water; 60.3 mg rATP; 200 mg yeast RNA (Sigma)), for 15 minutes before the addition of the 20  $\mu\text{l}$  of the kinased oligo. The filters were incubated at  $37^{\circ}\text{C}$  for at least one hour, and then washed 3 times with 50 ml of 6x SSC at  $37^{\circ}\text{C}$  for 10 minutes (low stringency wash). Filters were air dried, covered with Saran wrap and exposed overnight with Kodak X-AR film. - -

At page 212, please replace the paragraph beginning in line 7 with the following paragraph.

- - The fusion protein was shown to be catalytically active by incubation of the fd-tet-SNase phage ( $4 \times 10^9$  tetracyclin resistant colonies [TU]) with single stranded DNA (1  $\mu\text{g}$ ) for 1 hr at  $37^{\circ}\text{C}$  in the presence of  $\text{Ca}^{2+}$ , and analysis of the digest by agarose gel electrophoresis (Figure 42). Nuclease activity was not detected with the parent fd-CAT2 ( $2 \times 10^{10}$  TU) phage alone or after three rounds of PEG precipitation of mixtures of fd-CAT2 ( $2 \times 10^{10}$  TU) with SNase (0.7  $\mu\text{g}$ ). Thus the nuclease activity results from the display of the enzyme on the surface of the phage and not from co-precipitated or soluble SNase set free by degradation of the fusion protein. The nuclease activity of fd-tet-SNase (Figure 42) lies in the same order of magnitude, ( $2 \times 10^8$  TU and assuming three copies of SNase per TU) as an equimolar amount of SNase (0.03 ng or  $10^9$  particles), and like the authentic SNase was dependent on  $\text{Ca}^{2+}$ , since incubation with 40 mM  $\text{MgCl}_2$  and 25 mM EGTA blocked activity (not shown). - -

At page 216, please replace the paragraph beginning in line 14 and continued to page 217 with the following paragraph.

- - The following mutator strains (R. M. Schaaper & R.L. Dunn J. Mol. Biol. 262 1627-16270, 1987; R. M. Schaaper Proc. Natl. Acad. Sci. U.S.A. 85 8126-8130 1988) were used:

NR9232: ara, thi, mutD5-zaf13::Tn10, prolac, F'prolac

NR9670: ara, thi, azi, mutT1, leu::Tn10, prolac

NR9292: ara, thi, mutH101, prolac, F'prolac

NR9084: ara, thi, mutT1, azi, prolac, F'prolac<sup>+</sup>ΔM15 M15

NR9046: ara, thi, supE, rif, nalA, metB, argE(am),

prolac, F'prolac

were kind gifts of Dr. R. M. Schaaper (Department of Health & Human Services, N1H, PO Box 12233, Research Triangle Park, N.C. 27709)

NR9046mutD5: NR9046 mutD5::Tn10

NR9046mutT1: NR9046 mutT1::Tn10

were constructed by P1 transduction according to standard procedures. Mutator strains were transfected with fdCAT2scFvB18 or fdDOGKanscFvB18 and transfectants selected for antibiotic resistance. Transfectants were grown for 24h at 37°C before mutant phage was harvested by PEG precipitation. The mutant phage were selected on a 1ml NIP (4-hydroxy-3-iodo-5-nitrophenylacetic acid)-BSA-Sepharose affinity column (prepared according to the manufacturers instructions) prewashed with 200ml of PBS and blocked by 20ml MPBS. Phage were loaded on the column in 10ml MPBS and unbound material reapplied to ensure complete binding. The column was subsequently washed with 10ml of

MPBS and 500ml of PBS. Phage bound to the affinity matrix was eluted with 5 column volumes of 0.33 mM NIP-Cap (example 48). - -

At page 218, please replace the paragraph beginning in line 21 with the following paragraph.

- - For expression of soluble scFv fragments, transformants in E.coli HB2151 were grown at 37°C in 1 litre 2xTY, 0.2% glucose, 0.1mg/ml ampicillin to an OD600 of 1 and expression of soluble scFv fragments induced by adding IPTG to 1mM. Cultures were shaken at 30°C for 16h. - -

At page 220, please replace the paragraph beginning in line 21 and continued to page 221 with the following paragraph.

- - The scFv fragments from scFvB18 and the scFv fragments containing the glycine to serine and tyrosine to aspartate mutations respectively were expressed in solution (following transformation into E.coli HB2151 as in example 27) at 30°C. They showed no differences in the ELISA signals between wild-type B18 and the framework mutant. The signal obtained from the phage antibody with the Tyr mutated to aspartate in CDR3 of scFvB18 was about 10x stronger. Expression yields were found to be comparable as judged by Western blotting using an antiserum raised against g3p (as described above). Affinity measurements were performed using fluorescence quenching as described in example 23. Affinity measurement of affinity purified scFv fragments however showed scFvB18, and the scFvB18 (Gly->Ser) and scFvB18 (Tyr->Asp) mutants all to have a comparable affinity of 20nM for NIP-CAP. - -

At page 228, please replace the paragraph beginning in line 23 and continued to page 229 with the following paragraph.

- - Approximately 4µg of total RNA in 20ul water was heated at 65°C for 3 minutes, quenched on ice and added to a 30 ul reaction mixture resulting in a 50ul reaction mixture containing 140mM KCl, 50mM Tris, HCl (pH8.1 @ 42°C),

8mM MgCl<sub>2</sub>, 10mM DTT, 500uM deoxythymidine triphosphate 500 uM deoxycytosine triphosphate, 500 uM deoxyadenosine triphosphate and 500 uM deoxyguanosine triphosphate, 80 units of human placental RNase inhibitor and 10pmol of the appropriate Forward primer (HulgG1-4CH1FOR, HuIgMFOR, HuCKFOR, HuCLFOR). Two ul (50 units) of avian myeloblastosis virus (AMV) reverse transcriptase was added, the reaction incubated at 42°C for 1 hour, heated to 100°C for 3 minutes, quenched on ice and centrifuged for 5 minutes. - -

At page 229, please replace the paragraph beginning in line 11 and continued to page 230 with the following paragraph.

- - For the primary PCR amplifications, an equimolar mixture of the appropriate family based BACK and FORWARD primers was used. (See specific examples 40a and 40b given later in this example). A 50ul reaction mixture was prepared containing 5ul of the supernatant from the cDNA synthesis, 20 pmol total concentration of the FORWARD primers, 250 uM dNTPs, 50mM KCl, 100mM Tris. HCl (pH 8.3), 1.5 mM MgCl<sub>2</sub>, 175ug/ml BSA and 1ul (5 units) *Thermus aquaticus* (Taq) DNA polymerase (Cetus, Emeryville, CA). The reaction mixture was overlaid with paraffin oil and subjected to 30 cycles of amplification using a Techne thermal cycler. The cycle was 94°C for 1 minute (denaturation), 57°C for 1 minute (annealing) and 72°C for 1 minute (extension). The product was analyzed by running 5ul on a 2% agarose gel. The remainder was extracted twice with ether, twice with phenol/chloroform, ethanol precipitated and resuspended in 50ul of H<sub>2</sub>O. - -

At page 230, please replace the three paragraphs beginning in line 4 and continued to page 231 with the following three paragraphs.

- - To make the Fab linker DNA, 13 separate PCR reactions were performed using HulgG1-4CH1FOR and each of the reverse VK or V lambda oligonucleotides.

The template was approximately 1ng of pJM-1Fab D1.3 (fig.48) The PCR reaction reagents were as described above and the cycle was  $94^{\circ}\text{C}$ :1 min,  $45^{\circ}\text{C}$ :1min and  $72^{\circ}\text{C}$ :1 min. The linkers were analyzed on a 4% agarose gel, purified on a 2% agarose gel, eluted from the gel on a Spin-X column and ethanol precipitated.

#### E Assembly PCRs

- - For PCR assembly of a human Fab approximately 1ug of a primary heavy chain amplification and 1ug of a primary light chain amplification were mixed with approximately 250ng of the appropriate linker DNA in a PCR reaction mixture without primers and cycled 7 times ( $94^{\circ}\text{C}$ : 2 min,  $72^{\circ}\text{C}$ :2.5 min) to join the fragments. The reaction mixture was then amplified for 25 cycles ( $94^{\circ}\text{C}$ :1 mi,  $68^{\circ}\text{C}$  -  $72^{\circ}\text{C}$ :1 min,  $72^{\circ}\text{C}$ :2.5 min) after the addition of 20 pmol of the appropriate flanking BACK and FORWARD primers.

#### F Adding Restriction Sites

- - The assembled products were gel purified and reamplified for 25 cycles ( $94^{\circ}\text{C}$ :1 min,  $55^{\circ}\text{C}$ :1 min,  $72^{\circ}\text{C}$ : 25min) with the flanking oligonuceotides containing the appended restriction sites. PCR buffers and NTPs were as described previously.

At page 231, please replace the paragraph beginning in line 5 and continued to page 232 with the following paragraph.

- - a. PCR assembly of a Fab from a human hybridoma: the human monoclonal anti Rh-D cell lines Fog-1 (IgG-k) was derived from EBV transformation of the PBLs of a Rh-D negative blood donor immunized with Rh-D positive blood and has been previously described (Melamed, M.D., et al., J. Immunological Methods. 1987. 104:245) (Hughes-Jones N.C., et al., Biochem. J. 1990. 268:135) (Gorick,

B.D. et al., Vox. Sang. 1988. 55:165) Total RNA was prepared from approximately  $10^7$  hybridoma cells. First strand cDNA synthesis was performed as described above using the primers HuIgG1-4CH1FOR and HuCKFOR. Primary PCRs were performed for the VH-CH1 using a mixture of the 6 HuVHBACK primers and HuIgG1-4CG1FOR and for the VK-CK using a mixture of the 6 HuVKBACK primers and HuCKFOR. A Fab construct was assembled as described above, restricted with SfiI and NotI, gel purified and ligated into pJM-1Fab D1.3 restricted with SfiI and NotI. The ligation mixture was used to transform competent E.coli E.M.G. cells. Ninety-six clones were toothpicked into media in microtitre plate wells, grown to mid-log phase at  $30 \pm 0.5^\circ\text{C}$  and then expression of the Fab was induced by heat shocking at  $42 \pm 0.5^\circ\text{C}$  for 30 min followed by growing for 4 hours at  $37 \pm 0.5^\circ\text{C}$ . The ninety-six clones were then screened for anti-Rh-D activity as described below. - -

At page 232, please replace the paragraph beginning in line 26 and continued to pages 233 and 234 with the following paragraph.

- - Assay for anti-Rh-D activity and demonstration of specificity: A 5% (vol/vol) suspension of either Rh-D positive (OR2R2) or Rh-D negative (Orr) erythrocytes in phosphate buffered saline (PBS, pH 7.3) were incubated with a papain solution for 10 min at  $37 \pm 0.5^\circ\text{C}$ . The erythrocytes were washed three times in PBS and a 1% (vol/vol) suspension of erythrocytes was made up in PBS supplemented with 1% (vol/vol) of bovine serum albumin (BSA). Fifty  $\mu\text{l}$  of a papain treated erythrocyte suspension and 50  $\mu\text{l}$  of phage supernatant were placed in the wells of round bottom microtitre plates and the plates were placed on a Titertek plate shaker for 2 min. After 15 min incubation at  $37 \pm 0.5^\circ\text{C}$  100  $\mu\text{l}$  of PBS/BSA was added to each well. The plates were centrifuged at 200 g for 1 min and the



supernatant was discarded. The erythrocytes were resuspended in the remaining PBS/BSA and the Fab fragments were crosslinked by addition of the 9E10 monoclonal antibody (50ul a 1ug/ml solution in PBS/BSA) directed against the myc peptide tag (Ward, E.S., et al., Nature 1989. supra). The plates were placed at room temperature (RT) until sedimentation had occurred. Agglutination of erythrocytes caused a diffuse button of erythrocytes and the results were evaluated macroscopically. Specificity was confirmed with a standard prepanelized (as above) panel of 9 erythrocyte suspensions in PBS (all suspensions blood group O, 4 D positive and 5 D negative) known to have homozygous expression of all the clinically relevant erythrocyte blood group alloantigens. The number of copies of the D antigen on the D positive cells varied between 10,000 and 20,000 per erythrocyte depending on the Rh genotype. Briefly, 50 ul phage supernatant in PBS supplemented with 2% (vol/vol) skimmed milk was mixed with 50 ul of a 2% erythrocyte suspension in PBS in glass tubes and incubated for 15 min at 37°C. After one wash with PBS/BSA the erythrocytes were pelleted and resuspended in 50 ul donkey anti-human lambda light chain (Sigma L9527, diluted 1:40 in PBS/BSA). The tubes were centrifuged for 1 min at 200g and agglutination was read macroscopically using "tip and roll" method. - -

At page 241, please replace the paragraph beginning in line 9 and continued to page 242 with the following paragraph.

- - Twenty percent of the RNA, containing the genetic material from approximately  $2 \times 10^7$  B-cells, was used for cDNA preparation as described in example 40. Heavy chains originating from IgG and IgM antibodies were kept separate by priming cDNA synthesis with either an IgG specific primer (HuIgG1-4CH1FOR) or an IgM specific primer (HuIgMFOR). Aliquots of the cDNA was used to generate four separate scFv libraries (IgG-K, IgG-lambda, IgM-K and

IgM-lambda) as described in example 40. The resulting libraries were purified on 1.5% agarose, electroeluted and ethanol precipitated. For subsequent cloning, the K and lambda libraries were combined giving separate IgG and IgM libraries.

Cloning of the library: The purified scFv fragments (1-4ug) were digested with the restriction enzymes NotI and either SfiI or NcoI. After digestion, the fragments were extracted with phenol/chloroform, ethanol precipitated. The digested fragments were ligated into either SfiI-NotI or NcoI-NotI digested, agarose gel electrophoresis purified pHEN1 DNA (6ug) (see example 24), in a 100 µl ligation mix with 2,000 U T4 DNA ligase (New England Biolabs) overnight at room temperature. The ligation mix was purified by phenol extraction and ethanol precipitated. The ligated DNA was resuspended in 10 µl of water, and 2.5 µl samples were electroporated into E.coli TG1 (50 µl). Cells were grown in 1 ml SOC for 1 hr and then plated on 2 x TY medium with 100 µg/ml ampicillin and 1% glucose (AMP-GLU), in 243 x 243 mm dishes (Nunc). After overnight growth colonies were scraped off the plates into 10 ml 2 x TY containing AMP-GLU and 15% glycerol for storage at -70°C as a library stock. - -

At page 244, please replace the paragraph beginning in line 19 and continued to page 245 with the following paragraph.

- - Rescue of Phagemid libraries for enrichment experiments: To rescue phagemid particles from the library, 100 ml 2 x TY containing AMP-GLU (see example 42) was inoculated with  $10^9$  bacteria taken from the library (prepared in example 42) (approx. 10 µl) and grown for 1.5 hr, shaking at 37°C. Cells were spun down (IEC- centrifuge, 4 K, 15 min) and resuspended in 100 ml

prewarmed ( $37 \pm 0.5^\circ\text{C}$ ) 2 x TY- AMP (see example 41) medium,  $2 \times 10^{10}$  pfu of VCS-M13 (Stratagene) particles added and incubated 30 min at  $37 \pm 0.5^\circ\text{C}$  without shaking. Cells were then transferred to 900 ml 2 x TY containing ampicillin (100  $\mu\text{g/ml}$ ) and kanamycin (25  $\mu\text{g/ml}$ ) (AMP-KAN), and grown overnight, while shaking at  $37 \pm 0.5^\circ\text{C}$ . Phage particles were purified and concentrated by three PEG-precipitations (see materials and methods) and resuspended in PBS to  $10^{13}$  TU/ml (ampicillin resistant clones). - -

At page 245, please replace the paragraph beginning in line 8 with the following paragraph.

- - Enrichment for phOx:BSA binders by selection on tubes: For enrichment, a 75 x 12 mm Nunc-immunotube (Maxisorp; Cat. No. 4-44202) was coated with 4 ml phOx:BSA (1 mg/ml; 14 phOx per BSA in 50 mM  $\text{NaHCO}_3$  pH 9.6 buffer) overnight at room temperature. After washing three times with PBS, the tube was incubated for 2 hr at  $37 \pm 0.5^\circ\text{C}$  with PBS containing 2% Marvel (2% MPBS) for blocking. Following three PBS washes, phagemid particles ( $10^{13}$  TU) in 4 ml of 2% MPBS were added, incubated 30 min at room temperature on a rotating turntable and left for a further 1.5 hours. Tubes were then washed with 20 washes of PBS, 0.1% Tween 20 and 20 washes PBS (each washing step was performed by pouring buffer in and out immediately). Bound phage particles were eluted from the tube by adding 1 ml 100 mM triethylamine pH 11.5 and rotating for 15 min. The eluted material was immediately neutralised by adding 0.5 ml 1.0 M Tris-HCl, pH 7.4 and vortexed. Phage was stored at  $4 \pm 0.5^\circ\text{C}$ . - -

At page 247, please replace the paragraph beginning in line 18 and continued to page 248 with the following paragraph.

- - Clones resulting from reinfected and plated phage particles eluted after 4 rounds of enrichment, were inoculated into 150  $\mu$ l of 2 x TY-AMP-GLU in 96-well plates (cell wells, Nunclon), grown with shaking (250rpm) overnight at 37°C. A 96-well plate replicator ('plunger') was used to inoculate approximately 4  $\mu$ l of the overnight cultures on the master plate into 200  $\mu$ l fresh 2 x TY-AMP-GLU. After 1 hr, 50  $\mu$ l 2 x TY-AMP-GLU containing  $10^8$  pfu of VCS-M13 was added to each well, and the plate incubated at 37°C for 45 min, followed by shaking the plate at 37°C for 1 hr. Glucose was then removed by spinning down the cells (4K, 15 min), and aspirating the supernatant with a drawn out glass pasteur pipet. Cells were resuspended in 200  $\mu$ l 2 x TY-AMP-KAN (Kanamycin 50 ug/ml) and grown 20 hr, shaking 37°C. Unconcentrated supernatant containing phage was taken for analysis by ELISA. -

At page 251, please replace the paragraph beginning in line 1 and continued to page 252 with the following paragraph.

- - 100  $\mu$ l of bacterial stock of the IgM phagemid library prepared as described (example 42), containing  $5 \times 10^8$  bacteria, was used to inoculate 100mls of 2xTY medium containing 100 $\mu$ g/ml ampicillin, 2% glucose (TY/Amp/Glu). This was grown at 37°C for 2.5 hours. 10 mls of this culture was added to 90 mls of prewarmed TY/Amp/Glu and infection carried out by adding 10mls of a 200 fold concentrate of KO7 helper phage lacking gene 3 (M13KO7gIII $\Delta$  No.3) (example 34) and incubating for 1 hour at 37°C without shaking. Preparation of M13KO7gIII No.3 was as described in example 34. After centrifugation at 4,000 r.p.m. for 10 minutes the bacteria were resuspended in 100 mls of 2 x TY medium

containing 100 µg/ml ampicillin (with no glucose). Titration of the culture at this point revealed that there were  $1.9 \times 10^8$  infected bacteria as judged by their ability to grow on plates containing both ampicillin (100µg/ml) and kanamycin (50µg/ml). Incubation was continued for 1 hour with shaking before transferring to 2.5 litres of 2xTY medium containing 100µg/ml ampicillin, 50µg/ml kanamycin, contained in five 2.5 litre flasks. This culture was incubated for 16 hours and the supernatant prepared by centrifugation. (10-15 minutes at 10,000 r.p.m. in a Sorvall RC5B centrifuge at  $4 \pm 0.5^\circ \text{C}$ ). Phage particles were harvested by adding 1/5th volume of 20% polyethylene glycol, 2.5 M NaCl, standing at  $4 \pm 0.5^\circ \text{C}$  for 30 minutes and centrifuging as above. The resulting pellet was resuspended in 40mls of 10mM Tris, 0.1mM EDTA pH 7.4 and bacterial debris removed by centrifugation as above. The packaged phagemid preparation was then re-precipitated, collected as above and resuspended in 10mls of 10mM Tris, 0.1mM EDTA pH 7.4. The litre of this preparation was  $4.1 \times 10^{13}$  transducing units/ml (ampicillin resistance). - -

At page 252, please replace the paragraph beginning in line 9 with the following paragraph.

- - Tubes coated with OX-BSA were prepared as described in example 45 for panning the phagemid library from example 42. The rescued library was also panned against tubes coated with bovine thyroglobulin (Sigma). These were coated at a concentration of 1mg/ml thyroglobulin in 50mM NaHCO<sub>3</sub> pH9.6 at  $37 \pm 0.5^\circ \text{C}$ , overnight. Tubes were blocked with PBS containing 2% milk powder (PBS/M) and incubated with 1ml of the rescued phagemid library (the equivalent of 250mls of culture supernatant) mixed with 3mls of PBS/M for 3 hours. Washing, elution, neutralisation and infection were as described in example 45. - -

At page 254, please replace the paragraph beginning in line 9 and continued to page 255 with the following paragraph.

- - 40 colonies derived from the third round of panning against thyroglobulin (THYPAN3) were picked into a 96 well plate and grown overnight at 37°C in 200µl of TY/Amp/Glu. Similarly 48 colonies from two rounds and 48 colonies from three rounds of panning against OX-BSA were grown (OX-PAN2 and OX-PAN3). Polyclonal phage were prepared at the same time. Next day 5µl from each culture was transferred to 100µl of fresh prewarmed TY/Amp/Glu grown for 1.5 hours and M13KO7gIII No.3 added (2 x 10<sup>5</sup> infectious phage per well in 100µl of TY/Amp/Glu). ~~these~~ These were incubated for 1 hour at 37°C without shaking, centrifuged at 4,000 r.p.m. for 10 minutes, resuspended in 150µl of 2xTY medium containing 100µg/ml ampicillin and incubated for a further hour with shaking before adding to 2mls of medium containing 100µg/ml ampicillin, 50µg/ml kanamycin. After overnight growth the cultures were centrifuged at 4,000 r.p.m. for 10 minutes and the supernatants collected. ELISA plates used to screen THYPAN3 clones were coated at 37°C overnight with 200µg/ml thyroglobulin in 50mM NaHCO<sub>3</sub>pH9.6. Plates used for OXPAN2 and OXPAN3 were coated at 100µg/ml OX-BSA in PBS at 37°C overnight. - -

At page 256, please replace the paragraph beginning in line 4 with the following paragraph.

- - Selected clones (11 anti-thyroglobulin, 5 anti-OX-BSA) representing each of the different BstNI restriction digest patterns were assayed for binding to a panel of irrelevant antigens. ELISA plates were coated with antigen (100 µl/ml in 50 mM NaHCO<sub>3</sub>, pH 9.6) by overnight incubation at 37°C. The panel of antigens consisted of keyhole limpet haemocyanin, hen egg lysozyme, bovine

serum albumin, ovalbumin, cytochrome c, chymotrysinogen, trypsin inhibitor, GAP-D11 (glyceraldehyde-3-phosphate dehydrogenase), bovine thyroglobulin and oxazolone-BSA. Duplicate samples of phage supernatant (80  $\mu$ l + 20  $\mu$ l 5 x PBS, 10% milk powder) were added to each antigen and incubated for 1 hour at room temperature. ~~the~~ **The** ELISA was carried out as described in example 18. -

-

At page 259, please replace the paragraph beginning in line 4 with the following paragraph.

- - This revealed that the DNA had been successfully mutagenised as judged by the presence of bands in all four DNA sequencing tracks at the nucleotide positions encoding L91 and L92. This mutagenised single stranded DNA was subjected to a further round of mutagenesis as above using either mutL32 or mutH101 oligonucleotides. Mutagenesis with mutL32 gave rise to 71,000 clones (pool called D1.3L32) while mutH101 gave 102,000 clones (pool called D1.3H101). These clones were scraped into 15mls of 2xTY/20% glycerol. Single stranded DNA derived from each pool was sequenced with the oligonucleotides D1.3L40 and LINKSEQ1 respectively, as described above, and shown to be correctly randomised.  
D1.3L40:

5' CAG GAG CTG AGG AGA TTT TCC 3' **(SEQ ID NO: 78)**

LINKSEQ1:

5' TCC GCC TGA ACC GCC TCC ACC 3' **(SEQ ID NO: 79)**

At page 259, please replace the paragraph beginning in line 24 and continued to page 260 with the following paragraph.

- - 10-20 $\mu$ l of bacteria derived from each mutagenised pool (plate scrapes) was used to inoculate 5mls of TY/Glu/Amp. All bacterial growth was at 37 $\pm$ 0.5 $^{\circ}$ C. After 2-3 hours growth, 1ml was diluted in 5mls of prewarmed TY/Glu/Amp and

infected by addition of 0.5 mls of a 200 fold concentrate of the M13K07gIII  $\Delta$  No.3 preparation described in example 34. After 1 hour of infection the cultures were centrifuged at 4,000 r.p.m. for 10 minutes, resuspended in 2xTY, 100 $\mu$ g/ml ampicillin, incubated for a further hour, transferred to 500 mls of 2xTY medium containing 100  $\mu$ g/ml ampicillin, 50  $\mu$ g/ml kanamycin and grown for 16 hours. The remaining steps of phage preparation were as described in example 44. Phage were finally dissolved in 10mM Tris, 1mM EDTA pH7.4 at 1/100th the original culture volume. - -

At page 261, please replace the paragraph beginning in line 18 with the following paragraph.

- - 40 colonies derived from the third round of column purification on TEL-Sepharose were picked into a 96 well plate and grown overnight at 37 $\pm$ 0 $^{\circ}$ C in 200 $\mu$ l of TY/Amp/Glu. Phagemid particles were rescued and prepared for ELISA as described in example 18. ELISA plates were coated overnight at 37 $\pm$ 0 $^{\circ}$ C with hen egg lysozyme (HEL) or turkey egg lysozyme (TEL) at a concentration of 200 $\mu$ g/ml in 50mM NaHCO<sub>3</sub> pH9.6 ELISAs were carried out as described in example 18. - -

At page 271, please replace the paragraph beginning in line 12 and continued to page 272 with the following paragraph.

- - Chick gamma globulin (CGG, Sigma, Poole, UK) and Bovine serum albumen (BSA, Boehringer, Mannheim, Germany) were conjugated with NP-O-succinimide or NIP-caproate-O-succinimide (Cambridge Research Biochemicals, Northwich, UK) based on the method described by Brownstone (Brownstone, A., Mitchison, N.A. and Pitt-Rivers, R., Immunology 1966. 10: 465-492). The activated compounds were dissolved in dimethylformamide and added to proteins in 0.2 M sodium hydrogen carbonate. They were mixed with constant agitation



for 16 hours at 4°C and then dialysed against several changes of 0.2 M sodium hydrogen carbonate. They were finally dialysed into phosphate buffered saline (PBS). The conjugates made were NP<sub>12</sub>CGG, NIP<sub>10</sub>BSA. The NIP<sub>10</sub>BSA derivative was subsequently biotinylated using a biotinylation kit purchased from Amersham (Amersham International, Amersham, UK). - -

At page 272, please replace the paragraph beginning in line 8 with the following paragraph.

- - Seven days after immunization cells from the spleen were prepared as described by Galfre and Milstein (Galfre, G. and Milstein, C. Methods Enzymol. 1981. 73:3-46). Red cells were lysed with ammonium chloride (Boyle, W. Transplantation 1968.6:71) and when cell selection was performed dead cells were removed by the method described by von Boehmer and Shortman (von Boehmer, H. and Shortman, K, J. Immunol, Methods 1973:1:273). The cells were suspended in phosphate buffered saline (PBS), 1% Bovine serum albumen, 0.01% sodium azide; throughout all cell selection procedures the cells were kept at 4°C in this medium. - -

At page 272, please replace the paragraph beginning in line 22 and continued to page 273 with the following paragraph.

- - Biotinylated NIP-BSA was coupled to streptavidin coupled magnetic beads (Dynabeads M280 Streptavidin, Dynal, Oslo, Norway) by incubating 10<sup>8</sup> beads with 100µg of biotinylated protein for 1 hour, with occasional agitation, and then washing five times to remove unbound antigen. The coupled beads were stored at 4°C in medium until required. For selection of antigen binding cells the cells (2-4x10<sup>7</sup>/ml) were first incubated for 30 minutes with uncoupled beads, at a bead: cell ratio of 1:1, to examine the degree of non-specific binding. The beads were then separated by placing the tube in a magnetic device (MPC-E Dynal) for 3-5

minutes. The unbound cells were removed and then incubated with NIP-BSA coupled magnetic beads, at a bead:cell ratio of 0.1:1, for 60 minutes, with occasional agitation. The beads and rosetted cells were separated as described above. The beads were then resuspended in 1 ml of medium and the separation repeated; this process was repeated 5-7 times until no unbound cells could be detected when counted on a haemocytometer. - -

At page 274, please replace the paragraph beginning in line 22 and continued to page 275 with the following paragraph.

- - For screening single colonies were picked into individual wells of microtitre plates (Bibby) in 200µl 2 x TY/Ampicillin 100µg/ml/0.1% glucose and then incubated at 37°C for 5-6 hours with agitation, Isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma, Poole, UK) was then added to a final concentration of 1 mM and the incubation continued for a further 16 hours at 30°C before harvesting the supernatants. The wells of Falcon ELISA plates (Becton Dickenson, N.J., USA) were coated overnight at room temperature with NIP<sub>10</sub>-BSA (40µg/ml in PBS) and then blocked with 2% skimmed milk powder in PBS for 2 hours at room temperature. The bacterial supernatants were added and incubated at room temperature for 1 hour and then the plates were washed three times with PBS. Peroxidase conjugated-Goat anti-mouse lambda-chain (Southern Biotechnology, Birmingham, USA) was added and again incubated for 1 hour at room temperature before washing six times with PBS and then developing with 2,2'-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (Sigma, Poole, UK) as the peroxidase substrate. The optical density at 405nm was measured using a Thermomax microplate reader (Molecular Devices, Menlo Park, USA) after 30 minutes. Western blotting using the C-terminal myc tag as described in example 27. - -

At page 280, please replace the Footnotes beginning at page 280 and continued to page 281 with the following Footnotes.

- - Footnotes: <sup>a</sup>Approximately  $10^{12}$  phage with the stated ratio of pAb (D1.3) :  
FDTPs/Bs were applied to 1 ml lysozyme-sepharose columns, washed and eluted.  
<sup>b</sup>TG1 cells were infected with the eluted specific binding phage and plated onto  
TY-tet plates. After overnight incubation at  $30-37^{\circ}\text{C}$ , the plates were  
analysed by hybridisation to the  $^{32}\text{p}$ , labelled oligonucleotide VH1FOR (Ward et  
al op cit) which is specific to pAb D1.3. <sup>c</sup>Single colonies from overnight plates  
were grown, phage purified, and tested for lysozyme binding. <sup>d</sup>Enrichment was  
calculated from the oligonucleotide probing data. - -